

10/601,700

**AMENDMENTS TO THE SPECIFICATION:**

*Please amend the last paragraph on page 1, continuing to page 2, as follows:*

Loline alkaloids (LA; saturated 1-aminopyrrolizidine alkaloids with an ether bridge, Fig.1), are produced in a number of associations of grasses with endophytes of the genus *Epichloë* and their asexual descendants, *Neotyphodium* spp. In addition, LA are reported from the plants *Adenocarpus* spp. and *Argyreia mollis* of the families Fabaceae and Convolvulaceae, respectively. LA produced in grass-endophyte symbioses have strong insecticidal and feeding-deterrant properties (Riedell, et al., 1999, *J Entomol. Sci.* **26**: 122-129; Wilkinson et al., 2000, *Mol. Plant-Microbe Interact.* **13**: 1027-1033). Moreover, grasses infected by LA-producing endophytes, such as *Neotyphodium coenophialatum* and *N. uncinatum*, have greater tolerance to drought conditions (Arechavaleta et al., 1989, *Agron. J* **81**: 83-90; Bacon, 1993, *Agric. Ecos. Environ.* **44**: 123-141) than grasses infected by closely related endophytes, such as *N. lolii*, that do not produce LA (Barker et al., 1997, *Agric. Ecos. Environ.* **44**: 123-141; Cheplick et al., 2000, *Mycol. Res.* **97**: 1083-1092.). Growth suppression (allelopathy) of neighboring plants by meadow fescue (*Lolium pratense*) infected with *N. uncinatum* may indicate a potential for additional beneficial roles of these alkaloids in grass plant competitiveness and persistence.

*Please amend the 3rd paragraph on page 2 as follows:*

In one aspect, the present invention provides isolated nucleic acid compounds comprising at least a sequence identical or complementary to all or part of a coding sequence for the loline alkaloid biosynthetic gene cluster from *Neotyphodium* *Neotyphodium uncinatum* (SEQ ID NO. 15, and SEQ ID NO. 16). It appears that SEQ ID NO: 17 may be linked to the 5' end of SEQ ID NO: 16. Preferably, a part of said coding sequence is an open reading frame (ORF) selected from the group consisting of ORF1, ORF2, ORF3, ORF4, ORF5, ORF6, ORF7, ORF8, ORF9, ORF1', ORF2', ORF3', ORF4', ORF5', ORF6', ORF7', ORF8', ORF9' or ORF10'. More preferably, a part of said coding sequence is an ORF selected from the group consisting of ORF1, ORF2, ORF3, ORF4, ORF5, ORF6, ORF7, ORF8, ORF9, ORF1', ORF2', ORF3', ORF4', ORF5', ORF6', ORF7', and ORF8'.

10/601,700

*Please amend the 1st paragraph on page 5 as follows:*

Figure 3 is an autoradiograph showing expression of *lolA* and *lolC* genes in LA-producing (+) and suppressed (-) cultures. In each lane was loaded 0.5 µg of total cDNA synthesized from total RNA. cDNAs were probed with a mixture of a labeled 523 [[by]] bp fragment from *lolA* and a labeled 1427 [[by]] bp fragment from *lolC*. Identities of the hybridizing bands were confirmed in separate experiments with the individual probes (data not shown). Bottom panel shows expression of the *tub2* as a control. Molecular sizes (in kilobases) are indicated, and correspond to bands of a DNA-size marker (*Hind*III/*Eco*RI-cut λDNA) run in the same gel.

*Please amend the 1st paragraph on page 9 as follows:*

As used herein the term "mutation" refers to any change that alters a native coding sequence either by displacement, addition, deletion, insertion, cross-linking, or other destruction or substitution of one or more nucleotides of the native coding sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are also known [[in]] to those skilled in the art.

*Please amend the last paragraph on page 14, continuing to page 15 as follows:*

Other genes up-regulated during LA production that gave significant matches with known genes or sequences were a putative homing endonuclease, generally associated with unusual DNA splicing and incorporation events, and significant matches of cDNAs to sequences in the *N.* *Neurospora crassa* genome. However, for none of these genes do we currently have direct evidence for involvement in LA production. One sequence identified in four clones (P3, K8, C37, D5) was also detectable (by Southern blot) in at least one non-producer, *E. festucae* CBS 102477, but not in the LA producers *N. coenophialum* ATCC 90664 and *E. festucae* CBS 102475 (data not shown), suggesting that this gene is not involved in LA production.

*Please amend the last paragraph on page 15, continuing to page 16 as follows:*

**10/601,700**

Mycelium of *Neotyphodium uncinatum* (voucher specimen CBS 102646 at Centraalbureau Voor Schimmelcultures, Utrecht, The Netherlands) was isolated from grass leaf tissues [meadow fescue (*Lolium pratense* = *Festuca pratensis*), plant 167 in our plant collection] on potato dextrose agar as previously described (Blankenship *et al.*, 2001). The following procedures were carried out as described by Blankenship *et al.* (2001) with modifications. After 21 days of growth at 22 °C on PDA plates, 10 fungal colonies were transferred to, and homogenized in, 20 ml of LA-inducing medium (Blankenship *et al.*, 2001) with 15 mM asparagine and 20 mM sucrose as the nitrogen and carbon sources, respectively. Ten ml of the homogenate was added to a 500-ml Erlenmeyer flask with 100 ml of fresh LA-inducing medium, and the culture incubated at 22 °C with rotary shaking (100 rpm). After five days of growth, mycelium was harvested in 50-ml tubes (Falcon, distributed by Becton Dickinson Labware, Lincoln Park, NJ, USA) by centrifugation (2000 × g rcf), and the mycelium homogenized in 20 ml LA-inducing medium as described. To initiate main cultures for LA production, 1 ml of homogenized mycelium was added to 25 ml of LA-inducing medium and cultures were incubated as described above. To suppress LA production in cultures, but maintain growth conditions similar to the minimal medium, potato dextrose broth was added to give half-strength final concentration in the medium, and asparagine and sucrose were added to 7.5 mM and 10 mM final concentration, respectively. Except for this variation in medium composition, all growth conditions and source of inoculum for LA-suppressed cultures were the same as for LA-induced cultures. Cultures of *N. uncinatum* were grown under the conditions inducing or suppressing LA accumulation, and harvested during early accumulation when LA levels in the producing

10/601,700

medium were  $<20 \mu\text{g ml}^{-1}$ . (Levels in similar cultures later reached  $>1000 \mu\text{g ml}^{-1}$  in producing, but  $<10 \mu\text{g ml}^{-1}$  in suppressed cultures.)

*Please amend the last paragraph on page 18 continuing to page 19 as follows:*

cDNA synthesis and library construction were performed with the SMART<sup>TM</sup> cDNA Library Construction Kit (Clontech) according to the manufacturer's instructions. First-strand cDNA was synthesized with the same amount of RNA as used in the cDNA synthesis for the subtraction, and 2  $\mu\text{l}$  undiluted first-strand reaction was used as template to amplify the cDNA. The amplified cDNA was digested with *Sfi*I, size fractionated for removal of low-molecular-size ( $<0.1 \text{ kb}$ ) cDNA, and ligated into  $\lambda$ TriplEx2 vector (Clontech). cDNA ligated into vector was added to  $\lambda$  phage Gigapack III Gold packaging extract (Stratagene, La Jolla, CA, USA), and titered in *E. coli* strain XL1-Blue as specified by the manufacturer. For cDNA library amplification, overnight cultures of XL1-Blue were inoculated with an amount of packaged phage suspension to yield  $1.0 \times 10^5 \text{ pfu}$  per 150 mm plate (Falcon); in total, 15 plates were prepared, so the amplified library was derived from  $1.5 \times 10^6$  primary clones. After incubation overnight at 37 °C, to each plate was added 12 ml of  $\lambda$  dilution buffer (100 mM NaCl, 10 [[MM]] mM MgSO<sub>4</sub>, 35 mM Tris-HCl, pH 7.5, 0.01% gelatin), followed by 20 hr incubation at 4 °C. The phage suspensions were then titered for each plate. Since differences in titer between plates would affect representation of cDNA clones in the final amplified library, the appropriate volume of each suspension was determined so that, when combined, each plate contributed equally to the total number of pfu in the pooled library. After pooling, the titer of the amplified library was  $5.4 \times 10^9 \text{ pfu ml}^{-1}$ .

*Please amend the 2nd paragraph on page 19 as follows:*

Probes for Southern-blot, dot-blot and cDNA-based northern-blot hybridizations were labeled with DIG as described above. Total subtracted cDNA was labeled by using the primary PCR product in the subtraction as template and the nested PCR primers supplied with the PCR-Select<sup>TM</sup> cDNA Subtraction Kit (Clontech). Probe for *lolA* was a labeled 523 [[by]] bp fragment generated by PCR using primers *lolA*-5' (5'-GTCTGGCGAATTCTACAGACACG-3') (SEQ ID NO. 3) and *lolA*-3' (5'-

**10/601,700**

GATGGCCATGTGAGGAAAGAG-3') (SEQ ID NO. 4). A labeled 1427 [[by]] bp fragment of the *lolC* gene (see Results) was generated by PCR with primers *lolC*-5' (5'-CGGTGCGCGTCTTCTAAACTTGAC-3') (SEQ ID NO. 5) and *lolC*-3' (5'-GAATCTTCCGATGCAAGGCTTACG-3') (SEQ ID NO. 6).

**10/601,700*****Please amend Table 1 page 24 rows 2, 4, and 10, as follows:*****Table 1. Matches of subtracted cDNA clones with sequences in non-redundant (nr) and *Neurospora crassa* database BLAST searches.**

Clones <sup>1</sup>	Length in bp	nr matches, identity (%), and E values	Ns. crassa matches, identity (%), and E values
K8, C37, D5	468	[[-]]— <sup>2</sup>	—
B8, C5 <sup>3</sup>	633	<i>Sc. Pombe</i> <sup>4</sup> aspartate kinase gene, 24%, 5e-07	—
N17, C7	1521	[[-]]—	—
C2, D1	724	Krüppel-like C2H2 zinc finger transcription factors, 44%, 7e-08	various contigs (1.246; 1.392; 1.622; 1.686; 1.151), 35-52%, 4e-20 to 1e-05
C1, C3	283	—	—
E21	388	—	—
A6	370	—	—
A7	379	—	—
A8	554	[[-]]—	Contig 1.291 (57.61-57.83 kb), 56%, 7e-07
C8	430	<i>Sc. pombe</i> hypothetical protein, 42%, 2e-05	Contig 2.503 (15.96-16.19 kb), 38%, 7e-07
D2	472	—	—
D3	269	—	—
D4	694	rRNA intron-encoded homing endonuclease, 86%; 2e-11	various contigs (2.820; 2.798; 2.816; 2.793; 2.790; 2.943; 2.796, 2.843; 2.831; 2.957), 46 - 53%, 4e-10 to 3e-08
D6 <sup>5</sup>	374	homocysteine synthase/O-acetylhomoserine sulfhydrylase, 53%, 1e-22; related enzymes in methionine/cysteine biosynthesis, <1e-07); <i>RtxA</i> , enzyme in rhizobitoxine biosynthesis, 37%, 1e-10	Contig 2.65, 54%, 3e-24; Contig 2.688, 34%, 3e-11

***Please amend page 24, footnote 2 as follows:***<sup>2</sup> [[-]]— = No significant match.

10/601,700

Please amend Table 3 page 28, rows 5, and 8-9, as follows:

Table 3. LA phenotype of endophyte species and isolates used in this study. Indicated are the respective grass hosts which were used in the determination of the LA, and from which the endophytes in this study were originally isolated.

Species/isolate <sup>9</sup>	Grass host	Loline phenotype <sup>10</sup>	Reference <sup>11</sup>
1) <sup>12</sup> <i>Epichloë festucae</i> CBS 102477	<i>Festucae rubra</i>	—	1
2) <i>E. festucae</i> CBS 102475	N/A <sup>13</sup>	+	2
3) <i>E. typhina</i> 8	<i>Lolium perenne</i>	—	3
4) <i>Neotyphodium Neotyphodium</i> <i>aotearoae</i> CBS 109345	<i>Echinopogon ovatus</i> <i>Echinopogon ovatus</i>	+	4
5) <i>N. aotearoae</i> ATCC MYA-1231	<i>E. ovatus</i>	+	4
6) <i>N. australiense</i> CBS 109346	<i>E. ovatus</i>	—	4
7) <i>M. coenophiolum</i> <i>N. coenophialum</i> ATCC 90664	<i>Lolium arundinaceum</i>	+	3
8) <i>N. huernanum</i> <i>N. huernanum</i> ATCC 604040	<i>Festuca arizonica</i>	—	3
9) <i>N. inebrians</i> 818	<i>Achnatherum inebrians</i>	—	5
10) <i>N. lolii</i> 138	<i>L. perenne</i>	—	3
11) <i>N. melicicola</i> CBS 109342	<i>Melica decumbens</i>	—	4
12) <i>N. occultans</i> 999	<i>Lolium multiflorum</i>	+	6
13) <i>N. siegelii</i> ATCC 74483	<i>Lolium pretense</i>	+	7
14) <i>Neotyphodium</i> sp. 55	<i>Poa autumnalis</i>	+	3
15) <i>Neotyphodium</i> sp. 87	<i>Festuca paradoxa</i>	—	3
16) <i>Neotyphodium</i> sp. LpTG-2 Lp1	<i>L. perenne</i>	—	8
17) <i>Neotyphodium</i> sp. 269	<i>Hordeum bogdanii</i>	—	4
18) <i>Neotyphodium</i> sp. 270	<i>Hordeum brevisubulatum</i>	—	4
19) <i>Neotyphodium</i> sp. 361	<i>Hordelymus europaeus</i>	—	9
20) <i>Neotyphodium</i> sp. FaTG-3 Tf18	<i>L. arundinaceum</i>	+	4
21) <i>Neotyphodium</i> sp. FaTG-2 Tf14	<i>L. arundinaceum</i>	—	4
22) <i>Neotyphodium</i> sp. 4096	<i>Achnatherum robustum</i>	—	4
23) <i>N. uncinatum</i> CBS 102646	<i>L. pretense</i>	+	7

Please amend page 28, footnote 10 as follows:

<sup>10</sup> [=] "+" = loline-producing, [(-)] "—" = loline non-producing.

**10/601,700**

*Please amend page 29, footnote 15 as follows:*

<sup>15</sup> Reported is the sum of *N*-formyl and *N*-acetyl lolines in  $\mu\text{g g}^{-1}$  dry weight plant tissue. nd = not detected (limit of detection detection = 10  $\mu\text{g g}^{-1}$ ).

*Please amend the 8th paragraph on page 32 as follows:*

The *LOL2* gene cluster spans about a 16.4 kB region and consists of at least 8 ORFs. It appears that *LOL2* may include *lolF2* and *lolM* (SEQ ID NO: 17) linked to the 5' end of SEQ ID NO: 16, in which case the *LOL2* gene cluster would span about a 24kB region, consisting of 10 ORFs (i.e., ORF1' through ORF10'). ORFs of *LOL2* are indicated relative to nucleotide numbers annotated to SEQ ID NO: 16; mRNA sequences of each gene are given by joined exons determined by cDNA sequencing or predicted by the fgenesh (*Neurospora*) gene prediction program at "Softberry", <http://www.softberry.com/berry.phtml?topic=gfind>; gene orientations are indicated by "+" (forward strand) and "-" (reverse strand).

*Please amend the 2nd paragraph on page 34 as follows:*

#### **Example 9**

##### **Host-Vector System**

Identification and cloning of the loline alkaloid gene clusters is useful for the development of a host-vector system for the efficient recombination production of both novel and known alkaloids. The coding sequences which collectively encode a loline-type alkaloid gene cluster, including variants, hybrids, mutants, analogs or derivatives of the loline alkaloid gene cluster, can be inserted into one or more expression vectors, using methods known to those of skill in the art. The replacement gene cluster need not correspond to the complete native loline alkaloid gene cluster, but need only encode a functional gene cluster to catalyze production of an alkaloid.

*Please amend the 2nd paragraph on page 36 as follows:*

Further, the vectors, which collectively encode a replacement gene cluster can be inserted in to one or more host cell, using methods known to those of skill in the art. As

**10/601,700**

such, the present invention also provides host cells which have their naturally occurring gene substantially deleted, transformed with vectors encoding a replacement gene cluster or parts thereof, for the production of active alkaloids. The invention provides for the production of significant quantities of product at an appropriate stage of the growth cycle. The alkaloids so produced can be used as [[an]] insecticidal and feeding-deterrant agents to protect plants. The ability to recombinantly produce alkaloids also provides a powerful tool for characterizing biosynthetic enzymes and the mechanism of their actions.